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Title: BEAK MICROSTRUCTURE ANALYSIS AS A TOOL TO IDENTIFY POTENTIAL REARING STRESS FOR
Octopus vulgaris PARALARVAE

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Abstract: *Octopus vulgaris* is a viable candidate for commercial aquaculture, but rearing procedures might stress individuals and result in diminished growth and survival. The present study investigated the relationship between possible stress sources (tank transposition and siphoning) when rearing *O. vulgaris* paralarvae and the deposition pattern of growth increments in their beak microstructure. Light intensity at the facility was heterogeneous, and accounted for with an experimental design consisting of blocks without replicates. Growth and survival were estimated and possible effects of handling were tested for both parameters. Increments and stress marks were counted in 120 paralarval upper jaws (UJ), and the number of UJs with a mark on the day of stress application (day 8) was quantified. Differences in light intensity, diet quantity, and total number of marks in the UJ were also compared between treatments. Growth and survival were statistically similar between treatments, although the control treatment showed a tendency for higher survival rates. Age at first increment deposition coincided with day 1 of experiment, and a 1 increment.day⁻¹ deposition rate was validated for the experiment duration. The number of stress marks was significantly different between the control and other treatments, indicating that handling might cause stress and that marks can be used as a biomarker for stress, even though the occurrence of stress marks on day 8 was not significantly different. Light intensity and diet might have also been relevant stressors and confounded the results. The results herein presented are important for improving rearing conditions for *O. vulgaris* paralarvae.

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Highlights

- We investigated the relationship between the beak microstructure and stress events.
- Survival rates, though similar between treatments, seemed higher in control tanks.
- Growth increment deposition started at hatching and occurred on a daily basis.
- Stress marks were observed throughout the experiment and for all treatments.
- Factors other than handling (diet and light intensity) could be potential stressors.

**BEAK MICROSTRUCTURE ANALYSIS AS A TOOL TO IDENTIFY POTENTIAL
REARING STRESS FOR *Octopus vulgaris* PARALARVAE**

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Abstract

Octopus vulgaris is a viable candidate for commercial aquaculture, but rearing procedures might stress individuals and result in diminished growth and survival. The present study investigated the relationship between possible stress sources (tank transposition and siphoning) when rearing *O. vulgaris* paralarvae and the deposition pattern of growth increments in their beak microstructure. Light intensity at the facility was heterogeneous, and accounted for with an experimental design consisting of blocks without replicates. Growth and survival were estimated and possible effects of handling were tested for both parameters. Increments and stress marks were counted in 120 paralarval upper jaws (UJ), and the number of UJs with a mark on the day of stress application (day 8) was quantified. Differences in light intensity, diet quantity, and total number of marks in the UJ were also compared between treatments. Growth and survival were statistically similar between treatments, although the control treatment showed a tendency for higher survival rates. Age at first increment deposition coincided with day 1 of experiment, and a 1 increment.day⁻¹ deposition rate was validated for the experiment duration. The number of stress marks was significantly different between the control and other treatments, indicating that handling might cause stress and that marks can be used as a biomarker for stress, even though the occurrence of stress marks on day 8 was not significantly different. Light intensity and diet might have also been relevant stressors and confounded the results. The results herein presented are important for improving rearing conditions for *O. vulgaris* paralarvae.

Keywords: beak, biomarker, growth increments, *Octopus*, rearing, stress

Introduction

Octopus vulgaris Cuvier, 1797, or common octopus, is one of the best-known and most studied cephalopods in the world. The species meets some of the requirements to be considered as a viable candidate for commercial aquaculture, such as a short life cycle (Boyle & Rodhouse, 2005), fast growth (Mangold & Boletzky, 1973; Nixon, 1969), high food conversion rates (Mangold, 1983; Mangold & Boletzky, 1973; Navarro & Villanueva, 2003), easy adaptation to captivity (Iglesias et al., 2000), good acceptability of frozen and low-value food (Vaz-Pires et al., 2004), high reproductive rate and fecundity (Mangold, 1983), and high demand and market value (Vaz-Pires et al., 2004). Many culture programs have emerged in the past decades, mostly as scientific research at experimental scale, but some with commercial interests, such as seen in the 90's in Galicia, Spain (Iglesias et al., 2000; 2007). The paralarval stage, however, was and still is a bottleneck for commercial octopus aquaculture (Iglesias et al., 2000; 2007), and only a few rearing experiments have been able to reach the benthic stage (Carrasco et al., 2006; Iglesias et al., 2004; Itami et al., 1963; Villanueva, 1995). Octopus species which have benthic hatchlings instead of planktonic ones have been reared with greater success and, at times, with commercial interests, as is the case of *Octopus maya* in Mexico (Uriarte et al., 2011; Vidal et al., in press).

Captive individuals will likely display some level of stress, given that culture conditions differ from those of the natural habitat. Stress might also originate due to direct or indirect interaction with humans, but current knowledge about the physiological effects from stress caused by this handling is limited (Moltschaniwskyj et al., 2007; Pecl & Moltschaniwskyj, 1999). So far there are no validated biomarkers able to detect and quantify stress, although there are suggestions of appearance, behavior, and clinical signs of suffering in cephalopods (Andrews et al., 2013). The Directive 2010/63/EU has reinforced, among others, the refinement of culture experimental design and procedures. Although guidelines for cephalopod research in accordance with its proposal exist (e.g.: Andrews et al., 2013; Smith et al., 2013), the Directive itself does not provide guidelines for handling, probably due to the lack of scientific knowledge on the subjects of stress and pain (Sykes et al., 2012).

Cephalopod hard structures, such as the beak, develop by means of periodical increment depositions, and carry information on age and, possibly, on marked events occurring during ontogeny and on environmental influences on individuals (Canali et al.,

2011). It is possible that stressful events in culture conditions result in a distinct deposition pattern of these growth increments, which could be analyzed post-culture in order to identify stress sources and enable their elimination in future experiments. The pattern consists of bands of greater thickness than average, recorded as checks or darker increments, which can be distinguished among the (normal) periodical increments. These thicker bands, observed on squid statoliths (Villanueva, 2000) and octopus stylets (Barratt & Allcock, 2010; Hermosilla et al., 2010) and beaks (Canali et al., 2011), are known as stress marks, and inferred to be related to changes in life conditions (Perales-Raya et al., 2014).

The aim of the present study was to investigate the relationship between the beak microstructure of reared *O. vulgaris* paralarvae and possible stress events caused by handling treatments (siphoning and tank transposition). These were chosen as stress agents due to their common application in culture - siphoning for cleaning dead organic matter and tank transposition for procedures such as weighing and measuring individuals, etc. In order to do so, (i) paralarvae were reared for 15 days after hatching, with stress treatments applied in both acute and chronic way; (ii) growth (size and weight) and survival were compared between treatments in order to determine if stress had an overall effect on paralarval development; (iii) growth increments in the UJ of paralarvae were counted and related to the true age; (iv) the increment deposition pattern was analyzed to determine the presence or absence of stress marks, whether or not at the day of the stress event; and (v) results between treatments were compared to determine whether presence or absence of marks was indeed related to handling.

Material and Methods

Two types of handling stress were applied to reared paralarvae, namely tank transposition and siphoning. The two were performed concomitantly, with the control treatment being shared between them.

The experiment was performed according to the Spanish law 6/2013 based on the European Union directive on animal welfare (Directive 2010/63/EU on the protection of animals used for scientific purposes).

Octopus vulgaris broodstock

Wild *O. vulgaris* broodstock (700 to 2000 g) were captured in Tenerife, Spain, by artisanal fishermen and transferred to 1000-L circular fiberglass tanks at a ratio of two females per male, observing similar weights. Sex was determined by verifying the presence/absence of a hectocotylyzed arm. The tanks were part of a flow-through system with a 6 L.min⁻¹ flow rate, in which water entered the tank from its top (covered with a shady net) and left it through a 1 cm filter mesh positioned at the bottom. The natural photoperiod (from 10h light:14h dark to 11h light:13h dark) was maintained; mean water temperature was 20.69 ± 1.04 °C; salinity was 36.8 ± 0.14; and diet, given *ad libitum*, consisted of a mix of 50% frozen squid (*Loligo patagonicus*) and 50% prawns (*Parapenaeus longirostris*). PVC pipes and clay pots were placed inside the tanks to provide for dens.

In order to not disturb breeders, egg mass presence was checked once a week and, whenever it was observed, the remaining individuals were transferred to a different tank. Filter mesh was changed to a size of 363 µm when paralarvae were detected, and hatchlings were removed daily from the tank to ensure that there were always 1 day-old individuals on the next day. Hatchlings were randomly transferred to experimental tanks at a density of 1 individual.L⁻¹.

Paralarval rearing conditions

Paralarvae were reared in 1000-L fiberglass cylindrical tanks with black walls and bottom. Prey were offered daily between 08h30min and 09h30min, and consisted of 1-day old *Artemia* nauplii (Sep-Art EG INVE) enriched with Easy DHA Selco (INVE) for 24h (0.6 g.L⁻¹) and at a density of 0.05 individuals.mL⁻¹. Gentle and continuous aeration was supplied by an airstone. Tank renovation was of 86.4%.d⁻¹ (10 mL.s⁻¹), and a mesh size of 250 µm was used at the bottom of the tank to avoid loss of paralarvae. Water parameters were measured on day 8: temperature and dissolved oxygen with a Pro ODO dissolved meter (Yellow Springs, OH USA), ammonia (NH₃) and nitrite (NO₂) with TETRA test aquarium kits for NH₃/NH₄⁺ and NO₂⁻, and salinity with a hand refractometer (ATAGO S/mill-E). Experiments lasted for 15 days (after hatching). Upon termination, survival was estimated and 20 paralarvae were sampled from each tank (60 individuals per treatment), 10 for determining weight and 10 for determining size and subsequent beak analysis. Three different treatments were applied:

Tank transposition

The fragility of paralarvae to tank transposition was assessed by comparing the control treatment (C) with a tank transposition treatment (TT), in which the stress event was applied on day 8. Tanks were emptied out onto a 5-L volume bucket, where paralarvae remained concentrated for approximately 5 hours until the tanks were refilled and reset to the initial conditions and individuals could be transferred back into their respective initial tank.

Siphoning (chronic and acute)

The possible stress caused by siphoning (tank cleaning) was investigated based on three treatments: control (C), in which no siphoning was applied to tanks for the entire experiment; chronic siphoning (S_C), in which it was performed daily; and acute siphoning (S_A), in which it was performed on day 8 only (around the same time as for S_C).

Experimental Design (light intensity blocks)

The culture facilities provided poor natural illumination. An acrylic roof window increased (heterogeneously) sunlight intensity above tanks, and weather patterns were irregular and affected by day-light cycles, such that it was necessary to use an additional (artificial) light source to meet the paralarvae requirements. A fluorescent light was provided with a 12h light:12h dark cycle. Despite compensation, light intensity remained heterogeneous in the culture location. In order to account for these differences when distributing treatments between tanks, a randomized complete block design was applied (Fig. 1). Three light intensity blocks were identified, with higher (C_1 , S_{C1} , S_{A1} , and TT_1), intermediate (C_3 , S_{C3} , S_{A3} , and TT_3), and lower (C_2 , S_{C2} , S_{A2} , and TT_2) intensities (measured at the water surface), and each treatment was randomly placed in one tank per region, on a total of 12 tanks.

Measuring, weighing, and survival of paralarvae

Dorsal mantle length (DML), ventral mantle length (VML), and dry weight (DW) were recorded at the beginning (day 1) and end (day 15) of the experiment. DML and VML were measured to the nearest 0.01 mm with a Nikon Stereoscopic Zoom Microscope (SMZ-

10A-4x magnification) from fresh individuals anaesthetized with 2% ethanol. Measured paralarvae from day 15 were euthanized with chilled filtered (1 μ m) seawater and stored individually at -20°C for later beak extraction. Individual DW was obtained from a 0.01 mg precision balance (AT201, Mettler Toledo, Switzerland) after paralarvae were washed in distilled water and dried at 110°C for 24 h in an oven (Digiheat, SELECTA, Spain). The instantaneous specific growth rate (G, in % growth.day⁻¹) was calculated for DML, VML, and DW according to the formula $G = 100 * (\ln(Vt_2) - \ln(Vt_1)) / \Delta t$, where Vt_1 and Vt_2 refer to the individual variable (DML, VML, or DW) values at the beginning and end of experiment, respectively, and Δt is the experiment duration in days (Forsythe & Van Heukelem, 1987). The number of live paralarvae in each tank on day 15 was estimated and used to calculate the survival rate (S%) with the formula $S\% = (N_f / N_i) * 100$, where N_i and N_f are the initial and final number of paralarvae of each tank.

Beak extraction and microstructure analysis

Beaks were extracted from a total of 120 frozen paralarvae. Precision (very thin) needles were used to safely extract the beaks and a scalpel was used to cut the UJ in half under the binocular microscope. In the present study only the UJ of *O. vulgaris* paralarvae was used for identifying growth increments and stress marks because the LJ has very conspicuous teeth in the counting area (which makes it very difficult to identify and count increments), which is also shorter (anterior-posterior axis) than the UJ. When considering species other than *O. vulgaris*, however, it must be considered that the level of rostrum development varies according to species (for examples on squid and octopod species, see Franco-Santos & Vidal, 2014, and Franco-Santos et al., 2014, respectively) and that the structure might not be suitable for growth increment analysis.

Once extracted, increments and possible stress marks were observed in the UJ anterior colored (pigmented) region (ACR) with a Nikon DS-SM camera coupled to a Nikon AZ100 microscope with Differential Interference Contrast (DIC-Nomarski). Increments were counted on both left and right inner sides of the ACR. The counting area started at the rostral tip (first increment) and followed towards the posterior-most, darker (younger) increment (Fig. 2). The LJ was not used for microstructure analysis due to the difficulty in visualizing increments in its ACR. Several photos with the jaw sides in distinct positions (to account for differences in

illumination/shading) were taken to document the observed increment sequences (Fig. 3). Beaks were stored in individually labeled compartments and preserved in distilled water at 4°C to maintain the beak microstructure. Increments were independently counted (twice) for each side of the UJ by the same reader, and the counts were compared by a coefficient of variation (CV), a statistically rigorous method (Campana, 2001) used to assess reading precision. The coefficient is determined by the equation $CV = 100\% * (\sqrt{\sum(C_{ij}-C)^2}) / C$, in which C_{ij} is the i th age determined for the j th UJ side and C is the mean value for both counts (Campana, 2001). Besides being affected by reader ability, count precision is also influenced by the species and the structure for which age is estimated. It has been advised that studies should be carried out with a $CV < 7.6\%$ (Campana, 2001), so this value was adopted in the present study for sample discard.

Total number of increments from either of the two counts, when equal to experiment duration, was used to estimate the day at which a stress mark, in case it was observed, occurred. This back-calculated date was then compared with the day or period of stress applied by each treatment during rearing in order to confirm the deposition of thick and/or darker marks as a sign of stress in *O. vulgaris* paralarvae. Presence of stress marks was also analyzed for all experiment days for all individuals in order to evaluate a general deposition pattern and whether the pattern was related to treatments or to other factors detected during the experiment (i.e., differences in light intensity and diet quantity).

Statistical analysis

Normality and homocedasticity of data were checked with Shapiro-Wilk and Bartlett tests, respectively. Analyses were performed using R ver. 2.13.0 (Ihaka & Gentleman, 1993).

ANOVAs for blocks without replicates were run for (a) light intensity, diet (quantity), and total number of marks identified in the UJ, comparing both total and weekly differences in these parameters between treatments; (b) DML, G (DML), VML, G (VML), DW, G (DW), and survival, in order to test for significant differences between treatments in growth and survival; and (c) total number of UJs for which a stress mark corresponded to day 8 (when stress was applied). When ANOVA results were significant, the Tukey HSD (Honestly Significant Difference) post-hoc test was used to further identify differences. Paired t-tests were used for each treatment+light intensity combination (i.e., each tank) in order to identify

differences between (a) the first and second week in light intensity, diet, and number of marks on the UJ, and (b) the stress mark count on the left and right sides of the rostrum in the UJ.

Results

Culture parameters

Diet

Paralarvae were fed according to daily demand, so whenever food from the previous day was present, morning ration would be smaller. On day 4 of the experiment we had a logistic problem with the *Artemia* production, and the amount of food given to paralarvae decreased by more than 50% from the previous day and rose back to initial levels on the day after. Total prey offered to paralarvae in the first week was $44 \pm 10 (*10^3)$ *Artemia* nauplii for all tanks, and decreased in the second week to $29 \pm 6 (*10^3)$ nauplii for the C, $30 \pm 5 (*10^3)$ for the S_C, $29 \pm 7 (*10^3)$ for the S_A, and $31 \pm 7 (*10^3)$ for the TT treatments. There were no differences between treatments for the total (Fig. 4B) or weekly amounts, but all individual tanks had a significant decrease from week 1 to 2, with a higher significance for S_{A1} and C₃ (Tables 1 and 2).

Abiotic variables

Throughout the experiment, temperature was $19.9 \pm 0.4^\circ\text{C}$ and salinity was 36.8 ± 0.14 . On day 8, dissolved oxygen varied from 100.9 to 102.3%, ammonia was 0 mg.L^{-1} , and nitrite was $< 0.3 \text{ mg.L}^{-1}$.

Light intensity

As previously mentioned, the presence of two light sources (artificial and natural) resulted in a significant variability in light intensity, affecting the data obtained. In order to address this issue, light intensity was compared among different light blocks and treatments. Light intensity values throughout the entire experiment were (as expected) significantly different between the blocks (700 ± 188 , 505 ± 106 , and 397 ± 100 lux in the higher, intermediate, and lower intensity blocks, respectively), with differences observed between the

pairs of treatments C – TT, S_C – S_A, and S_C – TT (ANOVA and Tukey HSD tests, Table 1 and Fig. 4A). Changes in weather condition at the experiment location were noticed in the second week, at times causing lower light intensity values and often higher intensity variation during the day (difference between morning and afternoon readings). The mean and standard deviation values for the first and second weeks, respectively, were: 697 ± 153 lux and 701 ± 204 lux for the high light intensity region; 556 ± 91 lux and 376 ± 102 lux for the intermediate light intensity; and 441 ± 84 lux and 479 ± 104 lux for the lower light intensity. The analysis of individual tanks showed that the light intensity values were significantly different between weeks for C₂, S_{A2}, TT₂, and S_{A3} (Table 2).

Size, weight, and survival

Mean and standard deviation values for DML, VML, and DW; instantaneous specific growth rate (G) for DML, VML, and DW; and survival are provided in Table 3, while ANOVA results for differences in the above listed variables between treatments can be found in Table 4. All ANOVA tests for biometric measurements showed non-significant results.

Beak microstructure

General beak appearance was similar to that described for similar sized *O. vulgaris* paralarvae reared in Vigo, Spain (Franco-Santos et al., 2014), except for the absence of a small slit in the rostrum (whose protrusion is slightly less concave) in the LJ and a coloration pattern slightly more spread out in the hood region of both jaws.

Observations on embryonic beaks revealed that the first increment deposition coincided with the first day of the rearing experiment. Counting of increments was possible for all sampled individuals, although not for 100% of all right and left UJ sides. A total of 15 growth increments was observed in either or both sides of the UJ in 25, 29, 29, and 30 individuals (out of 30 sampled) from the C, TT, S_C, and S_A treatments, respectively. Of the seven (5.8% of the total 120) UJs which did not show 15 increments, five (4.2%) differed by one increment, one (0.8%) by two increments, and one (0.8%) by three increments. These values indicate that increments deposited daily during the experiments in the UJ ACR.

Coefficient of variation (CV) values between counts in the left and right sides of the UJ (Table 5) were all below the recommended value of 7.6% and no discards were necessary.

In the present study we have considered any increment that was darker than the average (Fig. 3) as a stress mark. Both daily increments and marks often joined with one another or disappeared (staggered growth phenomena – Lipinski, 1993). Overall, one or more stress marks were observed in the UJ of all extracted beaks. The number of stress marks found was significantly different between the control and all other treatments (which were similar among each other), both for the entire experiment duration and for the first and second weeks separately (Table 1, Fig. 4C). There was also a significant difference between the high and intermediate light intensity regions in the first week. All treatments within all blocks showed a similar number of stress marks on increment number 8, except for the C – S_A pair (Table 1, Fig. 4D). The paired t-tests indicated significant differences in the number of marks in the UJ between weeks for C₃, S_C (all), S_{A1} and S_{A2} treatments (Table 2), but showed no significant difference between the stress mark count of left and right sides of the UJ. Stress marks were found more frequently on increment number 5 (Table 6), present in 20, 26, and 30 UJs of the TT, S_C, and S_A treatments, respectively. A high number of stress marks were also recorded on days 4 and 6 to 9 (Table 6).

Discussion

Octopus vulgaris is one of the best known and most studied cephalopod species, especially when it comes to the field of aquaculture, and yet there are still many questions to be answered regarding viable rearing of the species. Cephalopod growth and survival is known to be influenced mainly by temperature levels and diet quality and quantity (a.o. Aguado Giménez & García García, 2002; Forsythe, 1993; Forsythe & Hanlon, 1988; Mangold & Boletzky, 1973; Moltschaniwskyj & Martínez, 1998), which is why most studies aim at understanding their effects on individuals in order to improve culture success (Vaz-Pires et al., 2004). Technology has made it possible to control temperatures, so nutrition is the current defining factor when it comes to paralarval mortality (Iglesias et al., 2007, 2013).

In the present study temperatures showed little variation and resembled the natural range, likely not influencing growth or increment deposition. Food was provided according to

tank demand, and tanks with prey left over in the morning received less food than tanks with little or no prey left from the day before. Food quantity provided to all tanks was similar for both weeks but decreased from the first to the second week. Although inappetence is commonly assumed as a distress indicator in vertebrates and could be used as a sign of suffering in cephalopods (Andrews et al., 2013), the fact that paralarvae ate less during the second week did not seem to constitute stress to paralarvae, as the number of stress marks in the UJs was not higher in the second week. Biometric (size and weight) and survival results were statistically similar, confirming the likelihood of diet not constituting a source of stress in the present study. The effect of a sharp, punctual decrease in diet quantity for a single day, however, might have been, and is discussed further in the text. Water quality parameters are also known to affect cultured animals, but it is likely that they did not influence measured variables in the present study, since salinity level was similar to the natural range, dissolved oxygen levels were appropriate, and nitrogenous compounds were either absent or present in very low levels.

Besides temperature, diet, and the above mentioned water quality parameters, other biotic and abiotic factors may have an effect on culture success, which highlights the need to determine standard conditions for optimum growth and survival (Estefanell et al., 2012; Uriarte et al., 2011). Few parameters have been examined, among which salinity (Villanueva et al., 2007), type and color of tanks (Sykes et al., 2011), and light type, photoperiod, and intensity (Sykes et al., 2013; Villanueva et al., 2007), but their effects on the formation of hard structures are practically unknown in cephalopods (Villanueva et al., 2003). High luminosity has been observed to positively contribute to female growth rate, maturation, and spawning (Iglesias et al., 2000). In the case of benthic hatchlings (up to 50 days-old) of *Sepia officinalis*, however, the use of low light intensity was more beneficial to growth and survival than the use of high intensity (Sykes et al., 2013). In the present study a slight decrease in light intensity was observed at times during the second week, although variation in intensity during the day (morning/afternoon) was higher in that period. The presence of the natural light source influenced the amount of change, such that the tanks farthest away from the roof window (C₂, S_{A2}, and TT₂) were the ones with significant differences between weeks. Another tank (S_{A3}) also showed a significant difference in light intensity between weeks, although its neighboring tanks did not, so this difference cannot be explained by its position within the culture facility. Marks on the UJ ACR were more frequent during the first week,

with significant weekly differences for S_{A2} . This specific tank also had a significant light difference (among others tanks), possibly indicating that high light intensities might be a stress source to paralarvae. Further studies should investigate light intensity alone as a stressor in order to verify this.

The initial DML of hatchlings was similar to that obtained by Villanueva (1995) and slightly smaller than that reported by Carrasco et al. (2006), while the initial DW was smaller than those of Villanueva et al. (2002), Iglesias et al. (2004), and Carrasco et al. (2006), perhaps due to differences in maternal feeding or in culture temperature (Márquez et al., 2013). DML and DW on day 15 were statistically similar for all treatments in the present study, and smaller than that obtained on day 10 by Carrasco et al. (2006). The instantaneous specific growth rate recorded by Villanueva (1995) for DML and VML on day 10 was much higher than those for day 15 in the present study, while that for DW on day 15 from Villanueva et al. (2002), which had a similar diet regime, was closer to that obtained herein on day 15. Survival rates on day 15 were statistically similar in the present study, and lower and higher than those found on day 20 by Carrasco et al. (2006) and Villanueva et al. (2002), respectively. It should be noted, nevertheless, that the survival rates recorded for the control tanks tended to be higher than those of the other treatments, so the effect of handling procedures on paralarval growth and survival should be further investigated.

It was possible to visualize growth increments and marks in both left and right internal sides of the UJ ACR, which did not differ from one another, contrary to what was noted for the increments in the lateral walls of *O. vulgaris* paralarvae (Hernández-López et al., 2001). Growth increments were also visible on both lateral walls in the UJ, but usually exceeded true age, possibly suggesting increment deposition during the embryonic stage, as seems to occur in *O. vulgaris* stylets (Barratt & Allcock, 2010). Stress marks, present in all UJs analyzed, were mostly short and never fully extended along the ACR, as previously observed for some lateral wall marks in *O. vulgaris* (Canali et al., 2011).

Increment visualization and interpretation is subjective, and depends on the ability to adjust the different focus and depths of field (Barratt & Allcock, 2010). Increment deposition in statoliths, beaks, and stylets has been suggested as an internally regulated process which occurs in the absence of feeding, vertical migrations, and/or temperature fluctuations (Dawe et al., 1985; Doubleday et al., 2006; Raya & Hernández-González, 1998, respectively), but it has also been suggested that changes in environmental parameters, such as daily sea surface

temperature, might contribute to the appearance of a stress mark (Perales-Raya et al., 2014). Mark detection is also associated with error, which could arise from bad sample preparation or defective sample, or even from the inability to distinguish a mark from the other increments (Canali et al., 2011). In the present study it was possible to estimate the reading precision, which was well below the recommended 7.6% value (Campana, 2001) and allowed for the use of 100% of the data collected.

Before one can estimate absolute age, it is necessary to determine the species specific age at first increment formation (Campana, 2001), otherwise number of increments could be under or overestimated and the ageing of individuals would be incorrect. Three studies on *Illex illecebrosus* statoliths, for example, have found distinct results, with increments being laid down before (Morris & Aldrich, 1985), at (Radtke, 1983), and after hatching (Dawe et al., 1985). A study on *Macroctopus maorum* stylets also suggested an underestimation of age due to non-validation of age at first increment formation (Doubleday et al., 2011). In the case of *O. vulgaris*, observations during the present study showed that there is no increment deposition in the UJ ACR during the embryonic stage, as also observed for hatchlings by Perales-Raya (unpublished results). It was possible, thus, to confirm hatching (day 1) as the age of first increment deposition for this structure in *O. vulgaris*.

Preliminary studies have hypothesized a daily increment deposition rate for *O. vulgaris* beaks (Perales-Raya et al., 2010; Raya & Hernández-González, 1998), which was validated for the lateral wall (UJ) in laboratory reared paralarvae (≤ 26 days old, Hernández-López et al., 2001), wild caught individuals (160-610 g, Canali et al., 2011), and for the entire age range (Perales-Raya, unpublished results). In the present study, 94.2% of UJs analyzed showed 15 increments corresponding to the 15 days of age, a much better rate than previously obtained with the lateral walls (48.1%, Hernández-López et al., 2001). The remaining 5.8% differed from the count by a maximum of 3 increments. Thus, the 1 increment.day⁻¹ deposition rate was also confirmed, validating the use of microstructure analysis on the UJ ACR as an ageing technique for laboratory reared paralarvae up to 15 days old.

In the present study it was not possible to observe a difference between treatments in the number of UJs which had a stress mark on the increment corresponding to the day (8) of stress application (except for the C and S_A treatments), indicating that stress marks might not be deposited at the very moment of stress. Nevertheless, the total number of marks identified was significantly different between the control and all other treatments for both weeks,

indicating that handling might constitute a source of stress and, perhaps, that marks be used as a biomarker for stress events in *O. vulgaris*. The fact that stress marks were present even in the UJs of paralarvae under the control treatment, however, strongly indicates that they were also subject to stress sources other than handling (also observed by Canali et al., 2011), perhaps light intensity, for which significant differences were found (between high and intermediate intensities) in total number of marks identified in the UJs. Another factor that may have contributed, at least punctually, to a high number of stress marks was diet quantity. As previously mentioned, on day 4 of the experiment the amount of food given to paralarvae decreased by more than 50% from the previous day and rose back to initial levels on the day after, and most stress marks identified in the present study were on increment (day) 5, the day after the 50% drop in food amount. This observation could indicate that marks are deposited after the stress event (*a posteriori*) but not necessarily on the very moment or day it occurs. In addition, when comparing the total number of marks per each increment (last row of Table 6), it is clear that the days with greatest values were 4 to 9, a time when paralarvae are running out of yolk reserve and must adapt to feeding only on captured prey (i.e., changing from endogenous (yolk) to exogenous (in this case *Artemia*) feeding), which could also be a source of stress. Studies on statoliths of *Stenoteuthis pteropus* (Arkhipkin & Mikheev, 1992) and stylets of *O. vulgaris* (Reis & Fernandes, 2002) have already hypothesized that formation of stress marks can be due to altered growth rates. In contrast to what was hypothesized earlier in this discussion, it seems that diet quantity might also have been a stress source in the present study. The effects of both light intensity and diet might have, thus, confounded the analysis and possibly altered the level of importance of handling as a stress source, but other uncontrolled factors could have also been relevant.

Future studies on stress agents in octopus rearing systems should better investigate culture parameters and variables in order to fully understand stress in paralarvae. An approach which could be used together with beak microstructure analysis is that of the genome-wide responses to stress (Zhang et al., 2012). Studies on the possible sources of stress in paralarval rearing and on stress biomarkers can aid in improving culture conditions and yields.

Conclusions

It the present study it was possible to visualize and count growth increments and stress marks in the UJ ACR of *Octopus vulgaris*. Observations showed that age at first increment formation coincides with hatching (day 1), and a 1 increment.day⁻¹ deposition rate was confirmed for paralarvae reared up to 15 days old. Growth and survival were not significantly affected by handling treatments, although there was a tendency for higher survival in the control treatment. Stress marks were not significantly present in the increment corresponding to the punctual application of stress (day 8). Comparing their presence in other days between treatments, however, indicated that handling procedures (siphoning and tank transposition) are a possible source of stress when rearing *O. vulgaris* paralarvae, although the results may have been confounded by light intensity and diet conditions in the present study. Marks in the UJ may, thus, be an effective biomarker to identify stress events when rearing *O. vulgaris* paralarvae. The results herein presented are important for improving rearing conditions and welfare of *O. vulgaris* paralarvae.

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Author contributions

Experimental design: RMF-S, CP-R, EA, DG. Performed the experiments: RMF-S. Data analysis: RMF-S, CP-R, EA, MDT. Wrote the manuscript: RMF-S. Revised the manuscript: RMF-S, CP-R, EA, MDT, DG.

Integrity of research and reporting

Ethical standards

The experiments described in the present study comply with the current laws of the country in which they were performed.

Conflict of interest

The authors declare that they have no conflict of interest.

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List of Figure Captions

Fig. 1 Experimental set-up, with indication of the natural (provided by the roof window) and
 artificial (provided by fluorescent bulbs) light sources and of the blocking design to account
 for different light intensities (horizontal, diagonal, and vertical stripes indicate the high,
 intermediate, and low intensity regions, respectively)

Fig. 2 Left and right sides of the interior portion of the upper jaw, with anterior and posterior
 jaw regions indicated. The circle indicates the anterior colored region where increment counts
 were performed, and the direction of increment count (from older to younger increments) is
 indicated by the arrow. Scale bar = 50µm

Fig. 3 Documenting process for upper jaws (UJ). The increment count is indicated, including
 the first and last increments and a possible stress mark (corresponding to increment/day 8). UJ
 regions are also indicated (hood, rostrum, and lateral wall). Scale bar = 50µm

Fig. 4 Plots resulting from ANOVAs for blocks without replicates comparing, between treatments and for the entire experiment duration, the (A) light intensity; (B) quantity of *Artemia* nauplii provided to tanks; (C) number of stress marks identified on the anterior colored region of the upper jaw (UJ); and (D) number of UJs which had a stress mark corresponding to day 8, when stress was applied to tanks. First, second (middle), and third lines in the box indicate the first, second, and third quartiles; whiskers indicate minimum and maximum values; and individual points represent outliers

Table 1

Table 1 Results for ANOVA for blocks without replicates ($F_{(\text{degrees of freedom})}$ and P) comparing total and weekly values of: number of stress marks identified in the anterior colored region (ACR) of the upper jaw (UJ), light, and diet (quantity); and for Tukey HSD test (P between pairs) identifying where differences lie. Significant values are underlined.

			Number of marks in the UJ ACR			Light			Diet			Number of UJs with a mark on increment 8
			1 st week	2 nd week	Total	1 st week	2 nd week	Total	1 st week	2 nd week	Total	
ANOVA	Treatments	$F_{(\text{df})}$	15.50 ₍₃₎	10.45 ₍₃₎	17.58 ₍₃₎	2.60 ₍₃₎	3.29 ₍₃₎	5.48 ₍₃₎	0.01 ₍₃₎	0.79 ₍₃₎	0.16 ₍₃₎	6.07 ₍₃₎
		P	<u>1.6 * 10⁻⁸</u>	<u>4.0 * 10⁻⁶</u>	<u>1.9 * 10⁻⁹</u>	0.06	<u>0.02</u>	<u>1.3 * 10⁻³</u>	1.00	0.50	0.92	<u>0.03</u>
	Blocks	$F_{(\text{df})}$	2.98 ₍₂₎	1.20 ₍₃₎	2.65 ₍₂₎	27.77 ₍₂₎	55.56 ₍₂₎	80.53 ₍₂₎	0.01 ₍₂₎	0.03 ₍₂₎	0.02 ₍₂₎	0.74 ₍₂₎
		P	<u>0.055</u>	0.31	0.08	<u>5.1 * 10⁻⁹</u>	<u>< 2.0 * 10⁻</u> 16	<u>< 2.0 * 10⁻</u> 16	0.99	0.97	0.98	0.52
Tukey HSD test	Treatment pairs	C – S _C	<u>8.0 * 10⁻⁷</u>	<u>0.02</u>	<u>3.8 * 10⁻⁶</u>	-	0.87	0.85	-	-	-	0.84
		C – S _A	<u>1.0 * 10⁻⁷</u>	<u>1.9 * 10⁻⁴</u>	<u>0.00</u>	-	0.53	0.27	-	-	-	<u>0.04</u>
		C – TT	<u>8.6 * 10⁻⁵</u>	<u>4.5 * 10⁻⁶</u>	<u>2.0 * 10⁻⁷</u>	-	0.17	<u>0.03</u>	-	-	-	0.08
		S _C – S _A	0.97	0.56	0.69	-	0.15	<u>0.05</u>	-	-	-	0.11
		S _C – TT	0.69	0.13	0.91	-	<u>0.03</u>	<u>2.0 * 10⁻³</u>	-	-	-	0.22
		S _A – TT	0.41	0.80	0.97	-	0.89	0.74	-	-	-	0.94
	Pairs of light intensity regions	High – Inter.	<u>0.05</u>	-	-	<u>4.1 * 10⁻⁴</u>	<u>0.00</u>	<u>0.00</u>	-	-	-	-
		High – Low	0.72	-	-	<u>0.00</u>	<u>0.00</u>	<u>0.00</u>	-	-	-	-
		Inter. – Low	0.24	-	-	<u>4.2 * 10⁻³</u>	<u>4.0 * 10⁻³</u>	<u>4.8 * 10⁻⁵</u>	-	-	-	-

Table 2 Results for paired t-tests ($t_{(df)}$ and P) per culture tank between the first and second weeks for the number of stress marks identified in the anterior colored region (ACR) of the upper jaw (UJ), light, and diet (quantity). Significant values are underlined.

		Number of marks in the UJ ACR	Light	Diet	Number of marks in the left x right sides
C_1	$t_{(df)}$	0.51 ₍₉₎	0.36 ₍₉₎	3.48 ₍₆₎	- 1.92 ₍₉₎
	P	0.62	0.72	<u>0.01</u>	0.08
C_2	$t_{(df)}$	1.00 ₍₉₎	2.38 ₍₉₎	3.48 ₍₆₎	- 1.12 ₍₉₎
	P	0.34	<u>0.04</u>	<u>0.01</u>	0.29
C_3	$t_{(df)}$	2.81 ₍₉₎	1.23 ₍₉₎	4.96 ₍₆₎	- 1.48 ₍₉₎
	P	<u>0.02</u>	0.25	<u>$2.5 * 10^{-3}$</u>	0.17
S_{C1}	$t_{(df)}$	3.67 ₍₉₎	- 0.01 ₍₉₎	3.48 ₍₆₎	- 0.32 ₍₉₎
	P	<u>$5.1 * 10^{-3}$</u>	0.99	<u>0.01</u>	0.75
S_{C2}	$t_{(df)}$	2.23 ₍₉₎	0.75 ₍₉₎	3.48 ₍₆₎	0.17 ₍₉₎
	P	<u>0.05</u>	0.47	<u>0.01</u>	0.87
S_{C3}	$t_{(df)}$	4.02 ₍₉₎	1.46 ₍₉₎	3.19 ₍₆₎	0.25 ₍₉₎
	P	<u>$3.0 * 10^{-3}$</u>	0.18	<u>0.02</u>	0.81
S_{A1}	$t_{(df)}$	2.41 ₍₉₎	- 0.36 ₍₉₎	4.42 ₍₆₎	- 0.75 ₍₉₎
	P	<u>0.04</u>	0.73	<u>$4.5 * 10^{-3}$</u>	0.47
S_{A2}	$t_{(df)}$	3.16 ₍₉₎	3.34 ₍₉₎	3.39 ₍₆₎	- 0.13 ₍₉₎
	P	<u>0.01</u>	<u>$8.7 * 10^{-3}$</u>	<u>0.01</u>	0.90
S_{A3}	$t_{(df)}$	1.81 ₍₉₎	2.36 ₍₉₎	3.48 ₍₆₎	0.96 ₍₉₎
	P	0.10	<u>0.04</u>	<u>0.01</u>	0.36
TT_1	$t_{(df)}$	0.0 ₍₉₎	- 0.26 ₍₉₎	3.19 ₍₆₎	0.09 ₍₉₎
	P	1.0	0.80	<u>0.02</u>	0.93
TT_2	$t_{(df)}$	0.25 ₍₉₎	3.05 ₍₉₎	3.19 ₍₆₎	- 1.77 ₍₉₎
	P	0.81	<u>0.01</u>	<u>0.02</u>	0.11
TT_3	$t_{(df)}$	1.40 ₍₉₎	1.80 ₍₉₎	3.19 ₍₆₎	- 1.16 ₍₉₎
	P	0.20	0.10	<u>0.02</u>	0.28

Table 3

Table 3 Mean \pm standard deviation values for dorsal mantle length (DML), ventral mantle length (VML), and dry weight (DW) at days 1 and 15, and for survival at day 15 are shown for each treatment. The instantaneous specific growth rate (G - $\% \cdot \text{day}^{-1}$) is also provided for DML, VML, and DW for the 15 days of experiment.

Treatment	DML (μm)			VML (μm)			DW (mg)			Survival (%)
	Day 1	Day 15	G ($\% \cdot \text{day}^{-1}$)	Day 1	Day 15	G ($\% \cdot \text{day}^{-1}$)	Day 1	Day 15	G ($\% \cdot \text{day}^{-1}$)	
C		2.47 ± 0.19	1.6		1.91 ± 0.19	1.7		0.49 ± 0.07	4.5	46.1 ± 8.2
S _C	1.95 ± 0.08	2.47 ± 0.14	1.6	1.48 ± 0.11	1.89 ± 0.12	1.6	0.25 ± 0.03	0.50 ± 0.06	4.6	25.4 ± 0.8
S _A		2.53 ± 0.15	1.8		1.95 ± 0.17	1.8		0.51 ± 0.07	4.7	33.2 ± 14.1
TT		2.49 ± 0.17	1.6		1.93 ± 0.21	1.8		0.50 ± 0.07	4.6	26.2 ± 15.3

Table 4 Results for ANOVA for blocks without replicates ($F_{(df)}$ and P) comparing values per treatment at day 15 for dorsal mantle length (DML) and ventral mantle length (VML); dry weight (DW); instantaneous specific growth rate (G) for DML, VML, and DW; and survival.

		DML	G - DML	VML	G - VML	DW	G - DW	Survival
Treatments	$F_{(df)}$	0.88 ₍₃₎	0.90 ₍₃₎	0.59 ₍₃₎	0.71 ₍₃₎	0.29 ₍₃₎	0.31 ₍₃₎	2.28 ₍₃₎
	P	0.46	0.49	0.62	0.58	0.83	0.82	0.18
Blocks	$F_{(df)}$	0.19 ₍₂₎	0.13 ₍₂₎	0.20 ₍₂₎	0.47 ₍₂₎	2.89 ₍₂₎	3.46 ₍₂₎	1.16 ₍₂₎
	P	0.82	0.88	0.82	0.65	0.06	0.10	0.38

Table 5 Mean coefficient of variation (CV) for the two increment counts for left and right sides of the upper jaw (UJ) for each light intensity region (block) of a treatment and for all blocks of a treatment. The confidence interval (CI) and the number of UJs analyzed (N) per block per treatment is also indicated.

Treatment	Block	Left side			Right side		
		CV	CI	N	CV	CI	N
C	High	2.71	1.38	10	2.55	1.81	10
	Medium	2.14	1.35	10	4.08	1.66	10
	Low	2.19	1.02	10	2.68	1.66	10
	All	2.35	1.22	30	3.10	1.67	30
S _C	High	5.88	1.42	10	7.15	1.81	10
	Medium	4.01	1.66	10	2.51	1.53	10
	Low	3.63	1.76	10	5.05	1.70	10
	All	4.51	1.60	30	4.90	1.77	30
S _A	High	3.00	1.26	10	2.08	0.97	10
	Medium	4.53	1.84	10	3.04	1.27	10
	Low	4.98	1.48	10	2.06	1.80	10
	All	4.17	1.52	30	2.39	1.35	30
TT	High	2.22	1.95	10	4.13	1.44	10
	Medium	5.22	2.02	10	1.96	0.91	10
	Low	5.66	2.03	10	1.58	1.29	10
	All	4.37	2.01	30	2.56	1.26	30

Table 6 Number of stress marks found per each increment number (i x) for all light intensities (Light) within a treatment (Treat)^a.

Treat	Light	i 1	i 2	i 3	i 4	i 5	i 6	i 7	i 8	i 9	i 10	i 11	i 12	i 13	i 14	i 15	Total
C	High	0	0	1	2	2	5	1	2	4	0	1	1	1	0	0	20
	Medium	0	0	1	4	5	2	1	1	2	1	1	3	0	1	0	22
	Low	0	3	4	4	5	5	4	5	4	0	0	0	0	0	0	34
	Total	0	3	6	10	12	12	6	8	10	1	2	4	1	1	0	76
S _C	High	0	1	6	2	10	6	5	4	3	1	2	0	1	1	0	42
	Medium	0	2	2	8	8	6	6	3	7	3	5	5	1	0	0	56
	Low	0	2	6	10	8	6	6	4	4	6	4	2	2	0	0	60
	Total	0	5	14	20	26	18	17	11	14	10	11	7	4	1	0	158
S _A	High	0	2	5	6	10	3	7	6	5	3	4	1	1	0	0	53
	Medium	0	1	8	9	10	3	3	8	6	5	3	1	0	0	0	57
	Low	0	0	7	8	10	4	7	7	6	5	6	3	1	0	0	64
	Total	0	3	20	23	30	10	17	21	17	13	13	5	2	0	0	174
TT	High	0	0	5	5	5	6	7	5	6	6	3	5	3	0	0	56
	Medium	0	0	2	4	9	9	7	8	8	7	4	3	0	0	0	61
	Low	0	0	4	6	6	5	9	6	3	6	4	2	1	0	0	52
	Total	0	0	11	15	20	20	23	19	17	19	11	10	4	0	0	169
All	Total	0	11	51	68	88	60	63	59	58	43	37	26	11	2	0	

^a Increment number corresponds to a day in the rearing experiment, such that increment 1 (i 1) = day 1, and so on, until increment 15 (i 15) = day 15. For each light intensity (for all treatments), a total of 10 upper jaws (UJs) were analyzed, so the total of one given increment for each treatment and for all the treatments represents a total out of 30 and 120 UJs, respectively.

Figure 1
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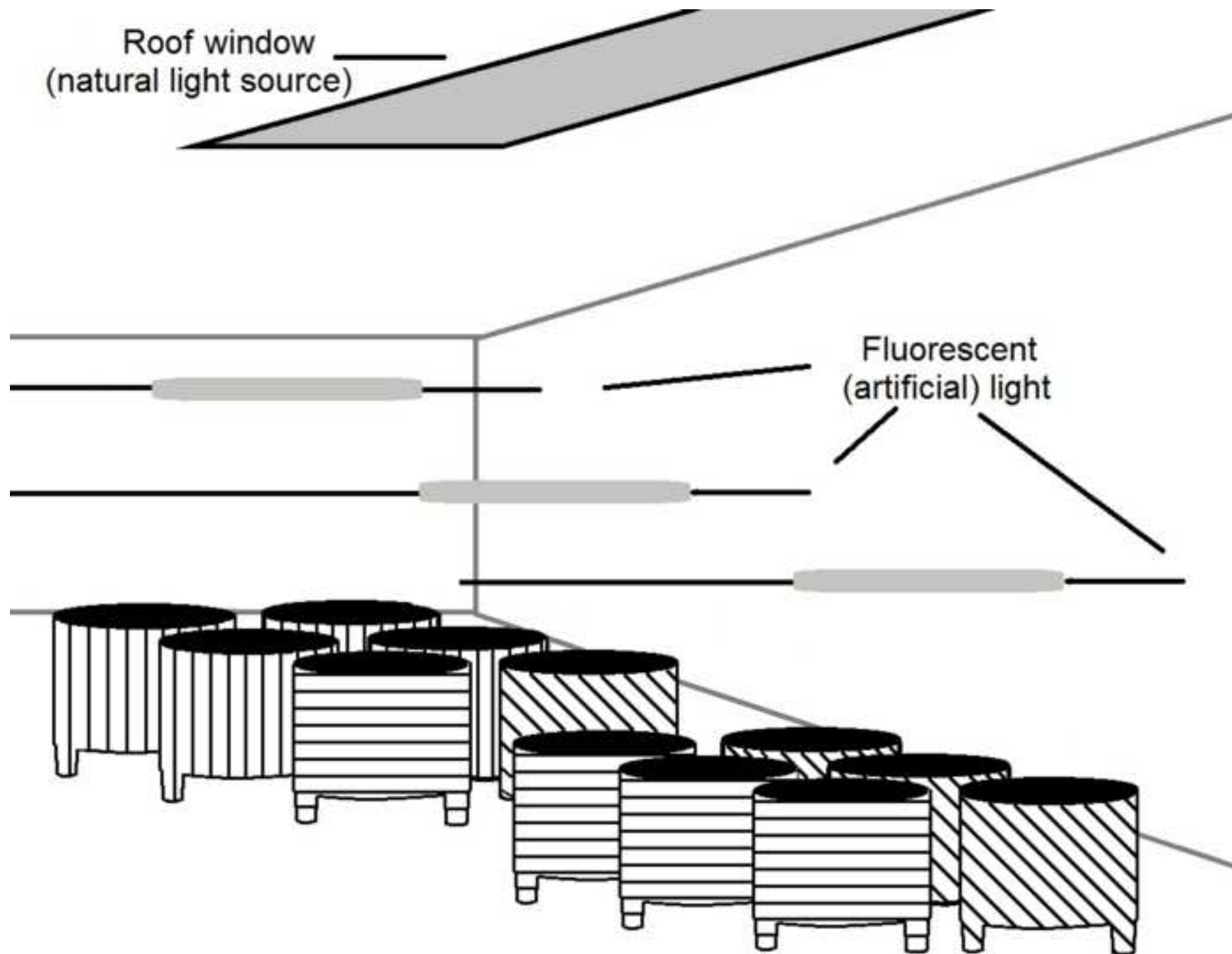


Figure 2
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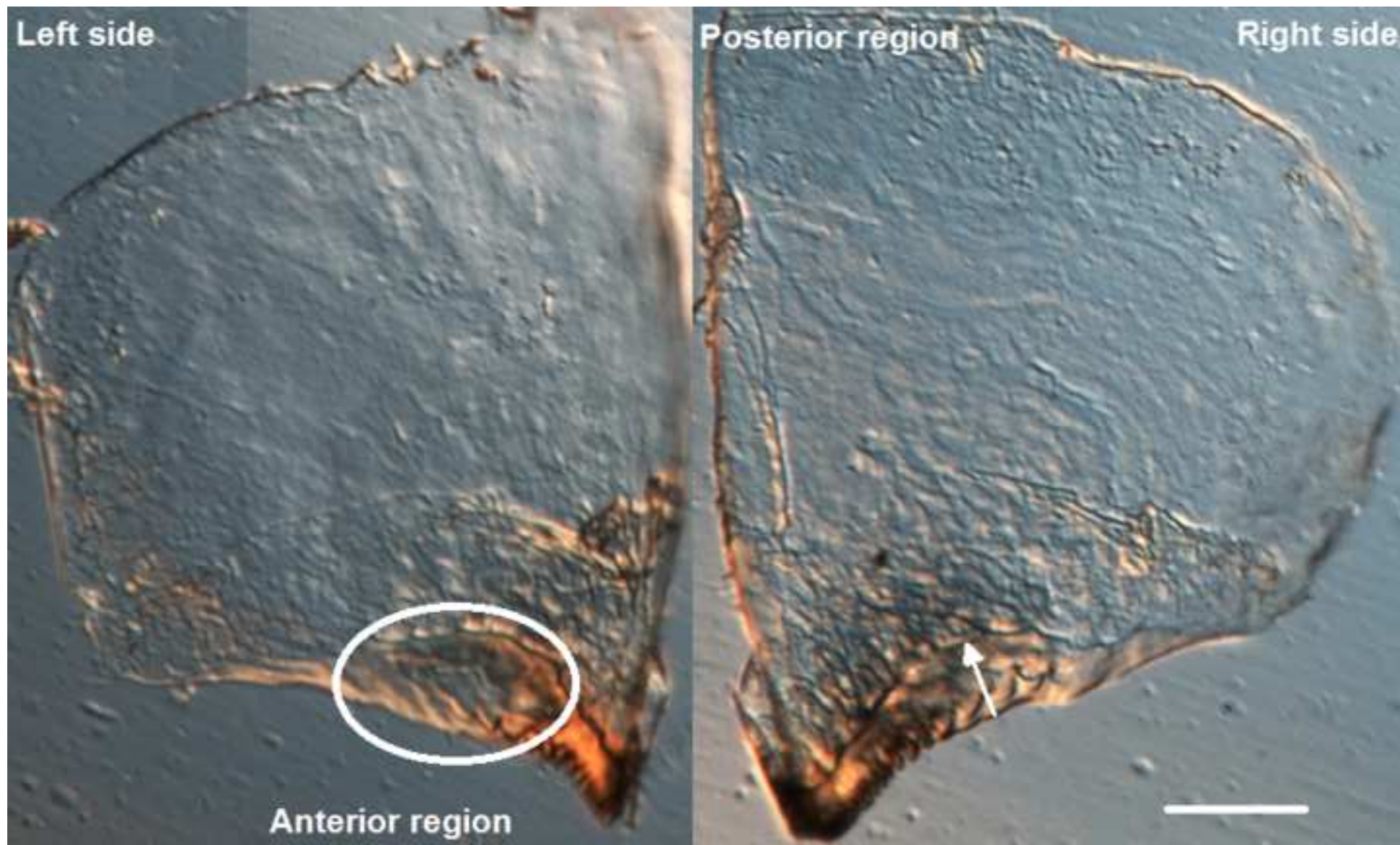


Figure 3
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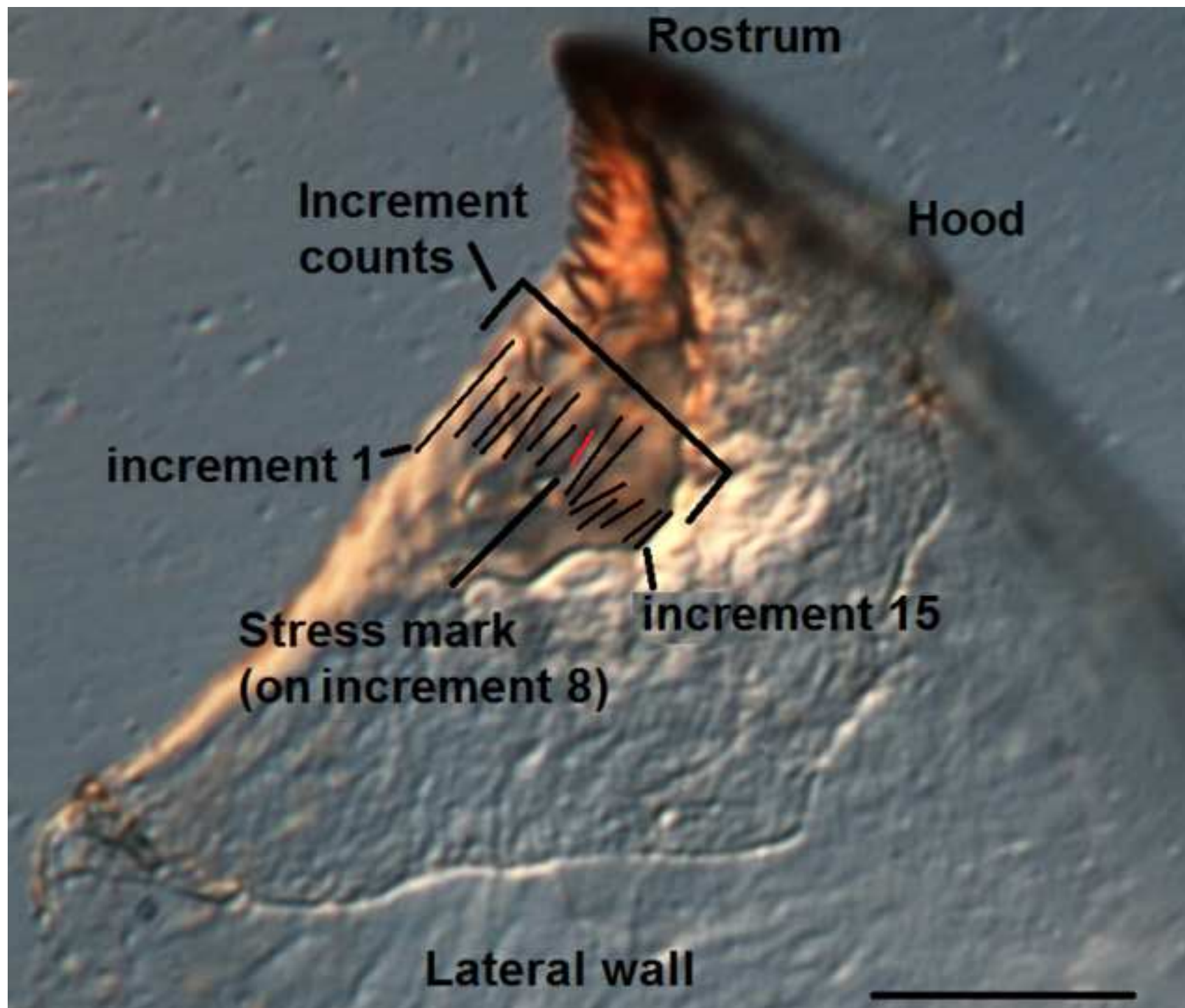


Figure 4
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